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(54) Title: METHOD FOR REDUCING THE IMMUNOGENICITY OF HETEROLOGOUS PROTEINS BY ELIMINATION OF T-CELL EPITOPES

(57) Abstract: The present invention provides a method for reducing the immunogenicity of a peptide or protein, which method comprises the steps of designing a series of overlapping test peptides each having an amino acid sequence that corresponds with part of the amino acid sequence of the peptide or protein of which the immunogenicity is to be reduced; identifying which of the test peptides of the series comprises one or more T-cell epitope(s); modifying the amino acid sequence of one or more of the test peptides comprising one or more T-cell epitope(s); repeating some of the steps with the modified test peptides until one or more of the T-cell epitopes originally comprised therein are significantly reduced or eliminated; modifying the amino acid sequence of the peptide or protein of which the immunogenicity is to be reduced according to the T-cell eliminating modifications made in the amino acid sequence of the test peptides to produce a modified peptide or protein. The invention also relates to the modified peptides and proteins thus obtained.

METHOD FOR REDUCING THE IMMUNOGENICITY OF HETEROLOGOUS PROTEINS BY ELIMINATION OF T-CELL EPITOPES

The present invention relates to a method for
5 reducing the immunogenicity of a peptide or protein by elimination of T-cell epitopes. The invention furthermore relates to a method for producing proteins and peptides having a reduced T-cell reactivity and to the peptides and proteins, in particular staphylokinase variants, thus
10 obtained. The invention also relates to the use of these modified peptides and proteins in therapy, diagnosis and prophylaxis.

An immune response involves, firstly, recognizing foreign material (pathogen/antigen) and
15 secondly, mounting a reaction to eliminate it. Broadly speaking, two types of reactions can be triggered; an innate or non-specific response and an adaptive or highly antigen-specific response. It is the latter reaction that improves with each successive encounter with the same
20 foreign material. The immune system remembers the foreign material, and deals with it more promptly, generating a life-long immunity following initial exposure.

Lymphocytes play a central role in all adaptive immune responses, since they specifically recognize
25 individual pathogens/antigens, whether they come from inside host cells or outside in the tissue fluids or blood. In fact, there are several different types of lymphocytes, but they fall into two basic categories, T-lymphocytes (or T-cells) and B-lymphocytes (or B-cells).
30 B-cells combat antigens and pathogens and their products by releasing specific antibodies.

T-cells can be divided in different types and display a wider range of activities. The so-called cytotoxic T-cells are responsible for the destruction of
35 host cells, which have become infected with viruses or other pathogens. T helper cells can interact with phagocytes and help to destruct pathogens. Other T helper

cells are involved in the control of B-lymphocyte development and antibody production.

Each B-cell is genetically programmed to encode a unique surface receptor specific for a particular
5 antigen. Having recognized its specific antigen, the B-cells can proliferate and differentiate into plasma cells, which produce large amounts of the receptor molecule in a soluble form, known as the antibody.

While B-lymphocytes recognize conformational
10 epitopes (3-D surface structure) on native antigens, T-cells recognize linear amino acid sequences (T-cell epitopes). But they can only do so, when the T-cell epitope is presented on the cell surface of other cells by the so-called human histocompatibility leucocyte
15 antigens (HLA) molecules. The antigen is not presented by the HLA molecules as intact proteins, but rather as processed peptides non-covalently bound in their peptide-binding groove. This processing and presentation can be done by specialized antigen presenting cells (APC), which
20 are capable of stimulating T-cells, or by infected cells, which then become a target for cytotoxic T-cells. The T-lymphocytes can recognize these HLA-peptide complexes by virtue of a specific receptor, called the T-cell receptor (TCR). Each TCR is unique, it recognizes a specific
25 peptide bound in the groove of a particular HLA-molecule. Upon recognition, the specific T-cell receives an activation signal, then a secondary signal is needed to induce a T-cell response, which includes proliferation and cytokine production.
30   If an individual encounters an antigen, and the immune system answers with a humoral response, a series of cellular and molecular interactions occur in an orderly sequence. The first interaction involves antigen-specific T-cells and APC. Its outcome largely dictates
35 the subsequent course of events. A sufficient number of T-helper cells needs to be triggered appropriately. Specific T-cells then respond with proliferation and cytokine secretion.

Subsequently, these activated T-helper cells interact with specific B-cells that present antigen to them. This B-T-cell contact in combination with the secreted cytokines, will activate the B-cells and

5 subsequently induce proliferation and differentiation into plasma cells. In turn, they produce antibodies, and depending on the T-cell signals they received, differentiate further by class switching, affinity maturation and the development of an immunological

10 memory.

Thus, for secondary or subsequent antigen encounter, a larger number of antigen-specific B-cells is present, making them the major type of APC, and importantly induce a shorter circuit in the immunological

15 response. However, if the T-helper cells are not triggered simultaneously, a form of immunological tolerance can develop, and no immunological events will follow.

Based on these considerations, the present

20 inventors contemplated that the elimination of functional T-cell epitopes will also have a great influence on the induction of a humoral response. If fewer or no specific T-cells are triggered, because specific peptides can no longer be efficiently presented, B-lymphocytes will be

25 hampered to mature and, consequently, the antibody production will be abrogated.

It is thus a first object of the present invention to provide a way of reducing the T-cell based immunogenicity of peptides and proteins.

30 This object is achieved by a method for reducing the immunogenicity of a peptide or protein, which method comprises the steps of:

a) designing a series of overlapping test peptides each having an amino acid sequence that

35 corresponds with part of the amino acid sequence of the peptide or protein of which the immunogenicity is to be reduced;

b) identifying which of the test peptides of the series comprises one or more T-cell epitope(s);

c) modifying the amino acid sequence of one or more of the test peptides comprising one or more T-cell epitope(s);

d) repeating step b) and optionally step c) with the modified test peptides until one or more of the T-cell epitopes originally comprised therein are significantly reduced or eliminated;

e) modifying the amino acid sequence of the peptide or protein the immunogenicity of which is to be reduced according to the T-cell eliminating modifications made in the amino acid sequence of the test peptides to produce a modified peptide or protein.

The above described method refers to the minimum amount of steps necessary. It is possible to make suitable modifications to test peptides in only one round. In case such modifications are sufficient this will appear in the repetition of step b). Such modifications can then be transferred to the peptide or protein of which the immunogenicity is to be reduced. It is however also possible to find upon repetition of step b) that further modifications are necessary. Step c) might refer also to more rounds of modification without an intermediate check for T-cell epitopes as in step b).

In addition to the above steps, there are further preferred steps that can be part of the method. It is for example possible to test the modified test peptides or the modified peptide or protein for their potential to activate T-cells and induce proliferation of T-lymphocytes as present in the circulation of humans (PBMC test). This represents an ex vivo situation and gives insight in the effect of the modifications on the in vivo immunogenicity.

Furthermore, it is possible to do the whole process of identification, modification (steps b) and c)) and optional PBMC testing all over again with the already modified peptide or protein.

The step of modifying the amino acid sequence of the peptide or protein of which the immunogenicity is to be reduced according to the T-cell eliminating modifications made in the amino acid sequence of the test
5 peptides can for example be performed by expression of a DNA sequence encoding the modified amino acid sequence in a suitable host.

The identification of peptides comprising a T-cell epitope can be performed in various ways. In one
10 embodiment use is made of a functional T-cell assay in which proliferation of one or more T-cell clones after stimulation of the T-cell clone(s) with one of the test peptides means that the said test peptide comprises a T-cell epitope. In a second embodiment use is made of a
15 functional T-cell assay in which proliferation of one or more T-lymphocytes as present in the circulation of humans (PBMC) after stimulation of the PBMC with one of the test peptides means that the said test peptide comprises a T-cell epitope. In these embodiments actual
20 peptides are used that are synthesized on the basis of the amino acid sequence of the peptide or protein of which the T-cell based immunogenicity is to be reduced.

Alternatively, the identification of peptides comprising a T-cell epitope is performed by determining
25 the interaction energy of the test peptide with the binding groove of one or more HLA-DR haplotypes by means of computer modeling, in which test peptide(s) are identified as comprising a T-cell epitope when according to their interaction energy they fit into the HLA-DR
30 binding groove. In this embodiment, the peptides are not actually synthesized but virtual peptides are used.

It is preferred according to the invention that the identification of peptides comprising a T-cell epitope is performed by a combination of the following
35 tests:

a) a functional T-cell assay in which proliferation of one or more T-cell clones after stimulation of the T-cell clone(s) with one of the test

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peptides means that the said test peptide comprises a
potential T-cell epitope, and/or

    b) a functional T-cell assay in which
proliferation of one or more T-lymphocytes as present in
the circulation of humans (PBMC) after stimulation of the
PBMC with one of the test peptides means that the said
test peptide comprises a T-cell epitope; and

    c) determining the interaction energy of the
said test peptide with the binding groove of one or more
HLA-DR haplotypes by means of computer modeling, in which
the test peptide is identified as comprising a potential
T-cell epitope when according to its interaction energy
it fits into the HLA-DR binding groove,
wherein a test peptide is considered to comprise a T-cell
epitope if it is identified in test a) and/or b) and c).

    In this embodiment, one test is a check for the
other. Only a T-cell assay can give information on the
functionality of the T-cell epitope. A peptide that
theoretically fits the HLA binding groove may not lead to
T-cell proliferation.

    The present invention thus deals with the
identification of functional human T-cell epitopes
(immunogenic regions) in proteins, preferably based on
the combined results of computer modeling of test
peptides in the HLA-DR binding groove and human T-cell
analysis. More specifically, the present invention
relates to a method calculating the interaction energy
between two proteins/peptides.

    Step d) of the above described method of the
invention deals with the re-evaluation of newly
introduced epitopes in the modified protein, preferably
by combining the results of computer modeling of the
modified test peptides in the HLA-DR binding groove and
human T-lymphocyte screening with the modified protein.

    The purpose of the method described above is
the reduction of the immunogenicity of foreign proteins
by elimination of the identified immunogenic regions. The
invention according to a further aspect thereof relates

to methods to remove the immunogenic regions by either
deletion or insertion of the coding sequences, or by
humanization of the coding sequences, or by _in vitro_
mutagenesis of the coding sequences to replace one or
5   more codons for wild type amino acids by a codon for
another residue, or by making the immunogenic regions
more sensitive for the proteolytic endosomal enzymes. The
latter type of modification can influence the manner in
which the T-cell epitope is presented on antigen
10 presenting cells. In addition, modification of
immunogenic regions to make them more sensitive for
proteolytic enzymes can result in the generation of
peptides that are too short to fit into and bind the HLA-
DR groove. It is known that 9 amino acids fit in the
15 groove, but that a minimum of 11 amino acids is necessary
for binding. Changing the position of the target sites
for endogenous proteolytic enzymes may lead _in vivo_ to
the production of shorter peptides that do no longer bind
the HLA-DR groove.
20   The present invention also relates to methods
for producing modified peptides and proteins of the
invention by preparing a DNA fragment encoding at least
the part of the structural information of the peptide or
protein that provides for its biological activity;
25 mutating the DNA fragment to eliminate therefrom the
coding information for one or more T-cell epitopes;
cloning the mutated DNA fragment in a suitable vector,
transforming or transfecting a suitable host cell with
the vector, and culturing the host cell under conditions
30 suitable for expressing the DNA fragment.
          More specifically, the invention provides a
method for producing a peptide or protein having a
reduced immunogenicity in comparison to wild type peptide
or protein, comprising the following steps:
35   a) providing a DNA sequence encoding at least
the amino acid sequence of the peptide or protein that
provides for the biological activity thereof;

b) identifying one or more of the T-cell epitopes comprised in the amino acid sequence of the wild type peptide or protein by:

    1) designing a series of overlapping test peptides each having an amino acid sequence that corresponds with part of the amino acid sequence of the peptide or protein;

    2) identifying which of the test peptides of the series comprises one or more T-cell epitope(s);

    3) modifying the amino acid sequence of one or more of the test peptides comprising one or more T-cell epitope(s);

    4) repeating step 2) and optionally step 3) with the modified test peptides until one or more of the T-cell epitopes originally comprised therein are significantly reduced or eliminated;

c) modifying the DNA sequence to introduce the modifications to the T-cell epitope(s) in the amino acid sequence encoded thereby;

d) introducing the DNA sequence into a host organism;

e) culturing the host organism under conditions allowing expression of the modified peptide or protein; and

f) isolating the peptide or protein having a reduced immunogenicity from the host organism.

In the above methods further steps may be incorporated as described above for the method for reducing the immunogenicity of a peptide or protein.

The peptides and proteins that can be produced according to the invention and that have a reduced T-cell based immunogenicity as compared to their wild type counterparts are novel and thus also part of this invention. The invention is applicable to reduce the T-cell based immunogenicity of all types of proteins and glycoproteins, either from human origin or originating from plants, micro-organisms or animals. Targets are also fusion proteins consisting in part of a protein from the

host in which the protein is produced and in part of the
desired protein. Other targets for the method of the
invention are humanized proteins.

When a protein or peptide is of human origin it
5 can still be immunogenic when administered to a human. An
example of this is the administration to a human
individual of a protein for which the individual is
deficient. The individual's body does not recognize the
protein as "self", even though it may be of human origin,
10 and will develop an immune response. Another example is
when a protein of human origin is no longer entirely
human, such as human interferon-α produced in E.coli
which is not glycosylated and found to be immunogenic, or
when proteins of human origin are modified to obtain
15 better properties, such as an increased solubility. In
particular, the invention is useful for lowering the T-
cell based immunogenicity of non-human proteins, such as
staphylokinase, streptokinase or antibodies from other
species or fragments thereof, or chimeric proteins, or
20 fusion-proteins, or de-novo proteins for the diagnostics
or treatment of human disease.

All such peptides and proteins that are in some
way or for some reason immunogenic when administered to a
human subject may be made less immunogenic by the method
25 of the invention. To illustrate this invention use is
made in the examples of staphylokinase. The invention is
however far more general applicable as has been explained
above and encompasses all proteins and peptides that are
made less immunogenic in this manner. According to a
30 particular embodiment of the proteins of the invention,
staphylokinase derivatives are provided that have a
reduced T-cell based immunogenicity as compared to wild
type staphylokinase.

While modifying a protein or peptide for
35 reducing its immunogenicity care should be taken that the
biological activity of the protein or peptide is not
severely decreased or even lost. It is thus preferred
that after producing a modified protein or peptide, its

biological activity is tested. Tests for determining the activity of the protein or peptide are usually available in the state of the art.

According to a further aspect thereof, the
5 invention relates to the modified peptide or protein for use in treatment, diagnosis or prophylaxis and to the use of a modified peptide or protein for the preparation of a pharmaceutical composition for treatment, diagnosis or prophylaxis of a human subject.

10   The present invention according to a further aspect thereof relates to pharmaceutical compositions comprising at least one of the less immunogenic derivatives according to the invention together with a suitable excipient, for diagnostics or treatment in

15 humans. Such compositions may be prepared by combining (e.g. mixing, dissolving etc.) the active compound with pharmaceutically acceptable excipients of neutral character (such as aqueous or non-aqueous solvents, stabilizers, emulsifiers, detergents, additives). The

20 concentration of the active ingredient in a therapeutic composition may vary widely, depending on the character of the disease and the mode of administration.

The present invention relates to so-called "T-cell based immunogenicity" which means that according to

25 the invention T-cell epitopes are identified, characterized and modified. The invention does not relate to general methods for the identification, production and use of staphylokinase derivatives showing a reduced antigenicity as compared to wild type staphylokinase,

30 based on the elimination of B-cell epitopes as previously described in US-5,695,754, US-5,951,980 and WO-9940198. These methods are totally distinct from and are not the subject of the present invention, although the antigenic regions (recognized by antibodies and B-cells) described

35 in these patent publications may (partly) overlap by serendipity with immunogenic regions involved in the T-cell response (T-cell recognition).

In a particular embodiment of the invention, the modified peptide or protein is staphylokinase. Staphylokinase, a protein produced by certain strains of <u>Staphylococcus aureus</u>, which was shown to have

5 fibrinolytic properties more than 5 decades ago appears to constitute a potent thrombolytic agent in patients with acute myocardial infarction. The <u>staphylokinase</u> gene has been cloned from the bacteriophages <u>sak</u>øC (Sako and Tsuchida, 1983) and <u>sak</u>42D (Behnke and Gerlach, 1987) as

10 well as from the genomic DNA (<u>sak</u>STAR) of a lysogenic <u>Staphylococcus aureus</u> strain (Collen et al., 1992). The <u>staphylokinase</u> gene encodes a protein of 163 amino acids, with amino acid 28 corresponding to the NH_2-terminal residue of full-length mature staphylokinase. The mature

15 protein sequence of the wild type variant SakSTAR is represented in Fig. 1. Only four nucleotide differences were found in the coding regions of the <u>sak</u>øC, <u>sak</u>42D and <u>sak</u>STAR genes, one of which constituted a silent mutation.

20   More in particular the invention relates to staphylokinase variants in which one or more of the following immunogenic regions are modified such that the T cell epitopes contained therein are eliminated:

        1-SSSFDKGKYKKGDDASY-17,
25   16-SYFEPTGPYLMVNVTGV-32,
        56-TKEKIEYYVEWALDATA-72,
        71-TAYKEFRVVELDPSAKI-87,
        106-ITEKGFVVPDLSEHIKN-122, and
        120-IKNPGFNLITKVVIEKK-136.

30   Furthermore, the invention relates to staphylokinase variants with reduced T-cell reactivity as compared to wild type staphylokinase, but retaining its biological activity, which variants have the amino acid sequence depicted in Figure 1 with one or more of the

35 following amino acid substitutions in one or more of the following immunogenic region:

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a) in the immunogenic region comprising SakSTAR
residues 16-32 the substitutions Y17L; F18L; F18E; E19D;
P20Y, Y24A; P20Y, Y24S; G22S; G22P, P23G; N28S;

b) in immunogenic region comprising SakSTAR
5 residues 71-82 the substitutions F76W, V79Y, D82A; R77A,
E80A; R77S, E80S; R77A, E80A, D82A; K74Q, R77S, E80S;
K74Q, R77S, E80S, D82G; K74Q, R77S, E80S, D82A; K74Q,
R77S, E80A, D82A; K74Q, R77A, E80S, D82A; K74Q, R77S,
E80S, D82S; V79Y, D82A;

c) in immunogenic region comprising SakSTAR
10 residues 106-122 the substitutions V112T; V112S;
D115N; E118S; H119A; H119S; V89L, L116Y; V89L, L116T;
V112T, H119S; V112T, H119A; V112T, E118S, H119S;

d) in immunogenic region comprising SakSTAR
15 residues 120-136, the substitution K130Y.

It is preferred that such staphylokinase
variants have a combination of one or more of the
mutations listed above, in particular one of the
following combination of mutations V112T, H119S, K130Y;
20 R77E, E134R; V29L, L127V; K74Q, R77E, E80S, D82S, E134R;
R77S, E80S, V112T, H119S; R77S, E80S, V112T, H119S,
K130Y; R77A, E80A, V112T, H119S; R77A, E80A, V112T,
H119S, K130Y; K74Q, R77S, E80S, V112T, H119S; K74Q, R77S,
E80S, V112T, H119S, K130Y; K74Q, R77S, E80S, D82S, V112T,
25 H119S; K74Q, R77S, E80S, D82S, V112T, H119S, K130Y.

Preferred staphylokinase variants are selected
from the group consisting of SakSTAR(P20Y, Y24A),
SakSTAR(P20Y, Y24S), SakSTAR(E19D), SakSTAR(Y17L),
SakSTAR(F18L), SakSTAR(F18E), SakSTAR(G22S),
30 SakSTAR(G22S, P23G), SakSTAR(N28S), and SakSTAR(V29L,
L127V), SakSTAR(R77E, E134R), SakSTAR(K74Q, R77E, E80S,
D82S, E134R), SakSTAR(K74Q, R77E, E80S, D82S,V112T,
H119S, E134R), SakSTAR(K74Q, R77E, E80S, D82S,V112T,
H119S, E134R, K130Y); SakSTAR(R77S, E80S, V112T, H119S),
35 sakSTAR(R77S, E80S, V112T, H119S, K130Y), SakSTAR(R77A,
E80A, V112T, H119S), SakSTAR(R77A, E80A, V112T, H119S,
K130Y), sakSTAR(K74Q, R77S, E80S, V112T, H119S),
SakSTAR(K74Q, R77S, E80S, V112T, H119S, K130Y),

sakSTAR(K74Q, R77S, E80S, D82S, V112T, H119S),

SakSTAR(K74Q, R77S, E80S, D82S, V112T, H119S, K130Y),

SakSTAR(V112T, H119A), SakSTAR(V112T, H119S),

SakSTAR(V112T, E118S, H119S), SakSTAR(V112T, H119S,

5   K130Y), SakSTAR(V112T), SakSTAR(V112S), SakSTAR(H119A),

SakSTAR(H119S), SakSTAR(V89L, L116Y), SakSTAR(V89L,

L116T), SakSTAR(V112T), SakSTAR(V112S), SakSTAR(D115N),

SakSTAR(E118S), SakSTAR(K130Y).

It was found that a staphylokinase variant in

10   which at least residue K130 of the wild type amino acid

sequence is replaced by a Y is particularly preferred.

To test whether the variants produced still

retain their thrombolytic activity a test can be used as

described in Collen et al., (1996b).

15   The staphylokinase variants can be used in

treatment, diagnosis or prophylaxis. The invention

furthermore relates to the use of a staphylokinase

variant for the preparation of a pharmaceutical

composition for treatment, diagnosis or prophylaxis of a

20 human subject and to a pharmaceutical composition

comprising a staphylokinase variant together with a

pharmaceutically acceptable carrier, diluents or

excipient.

The invention will be further elucidated in the

25 examples that follow, using staphylokinase as a model of

an immunogenic non-human protein with therapeutic

potential, but this example is not intended to limit the

scope of the invention, since several alternative

applications will be obvious for the persons skilled in

30 the art. Thus, the same methodology could be applied to

the de-immunization of other non-human or human proteins.

In the examples reference is made to the

following figures:

Figure 1. Primary sequence of staphylokinase

35 (SakSTAR). The amino acids are given in the one letter

code: A, alanine; D, aspartic acid; E, glutamic acid; F,

phenylalanine; G, glycine; H, histidine; I, isoleucine;

K, lysine; L, leucine; M, methionine; N, asparagine; P,

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proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophane; and Y, tyrosine.

Figure 2. Minimal epitopes analysis of a representative panel of region(71-87)-specific T-cell

5   clones. The T-cell clones were cultured with antigen presenting cells and SakSTAR peptide 71-TAYKEFRVVELDPSAKI-87, 15 peptides with single alanine substitutions in the region 71-87, and two 11-mer peptides (S1, S2), as indicated. If the proliferation of

10   a T-cell clone with the corresponding peptide was found comparable to that induced by the wild-type peptide (first column), a dark fill is plotted. A light gray fill indicates a significantly lower proliferation value compared to that with the same concentration of the

15   SakSTAR-(71-87) peptide, whereas a white box represents no detectable proliferation for the combination.

Figure 3. a: Modeling of 13 peptides (11-mer) encompassing the immunogenic region in SakSTAR. The non-bonded interaction energy (kcal/mol) of each peptide with

20   the HLA-DR1 structure is plotted. Longer bars denote a stronger HLA-DR1 binding. The two selected peptides, S1 and S2, are shown in black bars. b: Modeled structure of the representative peptide S2 in ribbon representation (Koradi et al., 1996). The S2 peptide is shown in light

25   gray, HLA-DR1 α- and ß-chain are color coded respectively in middle and dark gray. The side chain of ß-chain R71 interacting with peptide residue D82 is highlighted.

Figure 4. Alanine scanning experiment of the S1 (a,b,c) and S2 (d,e,f) peptide in the context of HLA-DR7.

30   **a,d:** The loss of interaction energy (kcal/mol) for each alanine mutant as compared to the wild type peptide is plotted. Longer bars mean that the corresponding peptide binds less well in the peptide-binding groove. **b,e:** Bar graph of the loss of contact area ($\mathring{A}^2$) for the same series

35   of peptides relative to the wild-type SakSTAR peptide. <u>c,f:</u> Plot of the solvent-accessible and buried surface area for each residue of the peptide bound in the groove. Positive values represent side chains exposed to the

15

solvent in the context of the complex, which are
potentially available for T-cell recognition. Negative
values indicate the extent of burying in the
corresponding HLA-DR pockets, numbered 1 to 9.

5   **Figure 5.** Proliferation responses of
staphylokinase (SakSTAR-myc)-primed PBMC to SakSTAR-myc,
the SakSTAR peptide 71-87 or peptide derivatives thereof.
PBMC from ten region-(71-87)-immunoreactive donors were
primed for SakSTAR-myc and re-stimulated with irradiated

10 CD3-depleted autologous PBMC and with no antigen,
SakSTAR-myc, wild type SakSTAR(71-87) peptide
TAYKEFRVVELDPSAKI, five single mutation(71-87)   peptides
(TAYAEFRVVELDPSAKI, TAYKAFRVVELDPSAKI, TAYKEFAVVELDPSAKI,
TAYKEFRVVALDPSAKI, TAYKEFRVVELAPSAKI), and four multiple

15 mutation(71-87) peptides (TAYKEFAVVALDPSAKI,
TAYKEFAVVALAPSAKI, TAYQEFSVVSLSPSAKI, TAYAAFAVVALAPSAKI).
The cellular response is plotted as stimulation index.
Each individual spot represents a cellular response from
a single donor to the indicated antigens/peptides.

20   **Figure 6.** Minimal sequence requirements of
epitope-specific T-cell clones. The T-cell clones were
cultured with APC and according to their specificity with
SakSTAR peptides 16-SYFEPTGPYLMVNVTGV-32 (16-26, and 23-
33), 56-TKEKIEYYVEWALDATA-72 (61-71), 71-

25 TAYKEFRVVELDPSAKI-87 (72-82, and 75-85), 106-
ITEKGFVVPDLSEHIKN-122 (110-120), or 120-
IKNPGFNLITKVVIEKK-136 (124-134) combined with single
alanine substitution peptides derived from the original
SakSTAR peptide. The number of analyzed clones is plotted

30 in the right column. The % of proliferating clones is
given for an alanine-substituted peptide at each residue.
A dark fill is plotted if more than 75% of the epitope-
specific T-cell clones proliferated when the indicated
residue was changed into an alanine. A medium gray fill

35 indicates that an alanine change affected less than 25%
of the epitope-specific T-cell clones in their
proliferative capacity. A light gray box indicates that
only 25-50% of the epitope-specific T-cell clones could

proliferate, and a white box represents a proliferative response of less than 25% of the epitope-specific T-cell clones. Thus, the whiter the box, the less T-cell clones proliferated to a peptide with an alanine substitution at

5 the indicated position. In summary, a white box indicate that the residue is important for HLA-DR binding or TCR recognition and will be the target for mutagenesis experiments.

10

EXAMPLES

EXAMPLE 1

Isolation of human staphylokinase-specific T-cell clones

Wild type recombinant staphylokinase (SakSTAR)

15 was expressed and purified as previously described (Collen et al., 1996a; Schlott et al., 1994), followed by an additional size exclusion chromatography step using a superdex 75 column (Pharmacia, Uppsala, Sweden). The purity of SakSTAR was determined to be greater than 98%,

20 as judged by SDS-PAGE analysis.

Normal healthy individuals were HLA-DR typed by the Inno-LiPa method (Buyse et al., 1993) (Innogenetics, Gent, Belgium) and their peripheral blood mononuclear cells (PBMC) isolated according to standard procedures

25 using Ficoll-Hypaque density gradient centrifugation. Ten healthy donors were selected representing >95% of the major HLA-DR haplotypes in the USA and Europe.

For the cloning of SakSTAR-specific T-cell clones, approximately 15×10^6 PBMC from each donor were

30 stimulated with an optimal concentration of SakSTAR for 7-9 days. The visible T-cell clones were harvested under the microscope, and individually re-stimulated in a 96-well flat-bottom plate with autologous PBMC as antigen presenting cells (APC). These wells were supplemented on

35 day 1 with 20U/ml recombinant human interleukin-2 (IL-2) (Boehringer Mannheim, Mannheim, Germany) and allowed to grow for 8-11 days. Re-stimulation was repeated every 8-11 days under the same conditions. After 3 to 6 passages,

autologous PBMC were replaced by autologous Epstein Barr
Virus (EBV)-immortalized B-cell lines as antigen
presenting cells.

In order to obtain autologous APC which could
5 be maintained for longer periods of time, EBV was
isolated from Marmoset B95-8 cells (ATCC#CRL1612)
according to standard centrifugation and filtration
procedures (Miller and Lipman, 1973).

Concentrated PBMC from the T-cell clone donors
10 were infected for 2 hours with EBV containing
supernatants. They were subsequently diluted and cultured
at 5.10^6 PBMC/ml, in RPMI1640 supplemented with 10% FCS,
10 μg/ml gentamycin and 1 μg/ml cyclosporin A. The B-
lymphocytes that were transformed with EBV could now be
15 maintained in culture and stored under liquid nitrogen.
These procedures resulted in the isolation of over 100
SakSTAR specific T-cell clones (CD3$^+$, CD4$^+$).

EXAMPLE 2
20 <u>Localization and characterization of immunogenic regions
in staphylokinase by functional T-cell assays</u>

To identify immunogenic regions on the SakSTAR
molecule, the isolated T-cell clones were tested for
their specificity using SakSTAR-derived peptides. In
25 total 25 peptides 17 residues in length, each overlapping
12 residues were synthesized using a fluorenylmethoxy-
carbonyl-protected amino acid coupling procedure
(Hiemstra et al., 1997). Each individual T-cell clone was
tested for its specificity by co-culturing T-cells,
30 Mitomycin C-treated autologous EBV-B-cells and antigen or
the appropriate peptide for 4 days. Subsequently, the T-
cell clones were pulsed for 24 hours with BrdU, harvested
and analyzed for their BrdU content. Proliferation after
stimulation with a specific peptide was found positive,
35 if the proliferation was at least three times the
background, revealing the specificity of that particular
T-cell clone.

These assays revealed the presence of 6
distinct immunogenic regions within the SakSTAR molecule.
The following peptides (SakSTAR residue number and
sequence) were found to be immunogenic:

5   1-SSSFDKGKYKKGDDASY-17,

       16-SYFEPTGPYLMVNVTGV-32,

       56-TKEKIEYYVEWALDATA-72,

       71-TAYKEFRVVELDPSAKI-87,

       106-ITEKGFVVPDLSEHIKN-122, and

10   120-IKNPGFNLITKVVIEKK-136.

EXAMPLE 3

<u>Localization and characterization of human T-cell</u>
<u>epitopes in staphylokinase by computer modeling of</u>
15 <u>peptides in the HLA-DR binding groove</u>

The total amino acid sequence of SakSTAR was
threaded as 11-mer sequences, shifting one residue at the
time, through the HLA-DR binding groove of the major
haplotypes, and the interaction energy of all these
20 combinations were calculated, using the dead-end
elimination theorem (DEE) (De Maeyer et al., 1997; Desmet
et al., 1992), which is embedded in the Brugel molecular
modeling package (Delhaise et al., 1984).

The energy function (Wodak et al., 1986) used,
25 comprises the usual terms for bond stretching, bond-angle
bending, a periodic function for the torsion-angles, a
Lennard-Jones potential for the nonbonded atom pairs, a
10-12 potential for hydrogen bonds, and a Coulombic
function for charged atoms. The dielectric constant has
30 been set to r_{ij}, the distance between the i and j atoms
(Warshel and Levitt, 1976). The energy parameters are
derived from the CHARMM library (Brooks et al., 1983).
All modeling uses the above energy function in the
presence of all explicit hydrogen atoms, the carboxylate
35 and imidazole groups were not protonated.

These calculations predicted that SakSTAR
contained several T-cell epitopes, which could fit in
most HLA-DR haplotypes.

19

EXAMPLE 4

Identification of T-cell epitopes in SakSTAR

　　　　Combining the specificity data of the isolated
T-cell clones with the predicted T-cell epitopes obtained
5 from computer modeling yielded largely overlapping
results. The identified SakSTAR T-cell epitopes are
summarized in Table I.

Table I Overview of the identified T-cell epitopes in
10 staphylokinase; SakSTAR

Position in Staphylokinase	Residues in Staphylokinase
6-17	KGKYKKGDDASY
16-26	SYFEPTGPYLM
23-33	PYLMVNVTGVD
43-53	HYVEFPIKPGT
61-71	EYYVEWALDAT
65-75	EWALDATAYKE
72-82	AYKEFRVVELD
75-85	EFRVVELDPSA
90-100	TYYDKNKKKEE
110-120	GFVVPDLSEHI
124-134	GFNLITKVVIE

25 The numbers represent the residue positions in the mature staphylokinase molecule, the amino
acids are represented by a single letter code.

The SakSTAR sequences 43-HYVEFPIKPGT-53 and
90-TYYDKNKKKEE-100 were calculated to fit in most HLA-DR
30 haplotypes, however, no T-cell clones were isolated that
proliferated to peptides which included these sequences.
The remaining 9 identified epitopes were found to be

functional, since epitope-specific T-cell clones were
isolated.

EXAMPLE 5

5 <u>Determination of the residue preference at each position
in the HLA-DR peptide-binding groove</u>

As described above computer modeling allows the
identification of amino acid sequences in a protein with
good interaction energy with the binding groove of the
10 HLA-DR molecule, which implies T-cell stimulating
capability and therefore immunogenicity.

The same computer technique may be used to
model sequences that are less favorable for binding at
each position in the peptide-binding groove. The DEE
15 implementation allows the calculation of the interaction
energy of the complete enumeration of all sequence
combinations for a 9-mer peptide bound in the groove.
From these results, the skilled person will be able to
extract an interaction preference matrix with the
20 calculation method as described in Example 3. This matrix
contains for each amino acid at each position in the
peptide the interaction energy with the HLA-DR molecule.
This interaction energy is interpreted as the likelihood
for an amino acid to be found in a binding peptide.

25 Therefore, less well binding residues may
possibly lower the affinity or even disrupt the binding
of the peptide in the HLA molecule. This matrix was
calculated for each HLA-DR haplotype. If the DR
restriction for an immunogenic region is known, these
30 tables can be consulted to design alternate sequences,
which bind less well in the groove, but do not interfere
with the 3-D folding of the protein. Since the different
DR matrices share a common pattern, a generalized matrix
can be assembled. In case the DR restriction is not
35 known, this generalized matrix can be consulted.

EXAMPLE 6

Identification of critical residues in an immunogenic
region by screening with a T-cell clone library
                Interestingly, the peptide with sequence
5   71-TAYKEFRVVELDPSAKI-87 of SakSTAR stimulated T-cell
    clones isolated from 9 of 10 donors, suggesting a
    promiscuous character. In order to define the minimal
    epitope for each individual T-cell clone, SakSTAR
    region(71-87)-peptide derivatives, each one with a
10 different single alanine substitution were used in T-cell
    proliferation assays. A representative panel is shown in
    Fig. 2.
                Alanine substitution at positions F76, R77,
    V78, or E80, abolished proliferation of the majority of
15 the region(71-87)-specific T-cell clones. Side chain
    removal by mutation into an alanine residue of K74 or D82
    affected the proliferative capacity of approximately half
    of the region(71-87)-specific T-cell clones, whereas a
    minority of them was found sensitive for an alanine
20 substitution of residues Y73, E75, V79, L81 or P83.

EXAMPLE 7

Analysis of an immunogenic region by computer analysis
                T-lymphocyte proliferation requires both
25 appropriate peptide presentation and T-cell receptor
    recognition. Consequently, T-cell assays alone cannot
    discriminate between HLA-DR-binding and receptor-facing
    residues within a peptide. To unravel the molecular basis
    of this ambiguity, a computer modeling approach was used
30 starting from available HLA-DR1 X-ray coordinates (Brown
    et al., 1993). The extended immunogenic SakSTAR sequence
    67-ALDATAYKEFRVVELDPSAKIEV-89 was threaded as 11-mer
    peptides, each shifted by a single residue, through the
    HLA-DR1 peptide-binding groove. The main-chain atoms of
35 both HLA-DR and the peptide were kept fixed, while all
    side chains were placed in the global minimum energy
    conformation using the dead-end elimination theorem
    (Desmet et al., 1992). Modeling in the context of the

other haplotypes, yielded essentially the same results. The interaction energy for each peptide with the groove was calculated, and is represented in Fig.3a.

Two strong binding sequences AYKEFRVVELD (S1; SakSTAR residues 72-82) and EFRVVELDPSA (S2; SakSTAR residues 75-85) were identified (Fig.3a; black bars), placing the second residue of both sequences in HLA-DR pocket 1. A typical model of the S2 peptide within the binding groove is shown in Fig.3b.

The extent of both solvent exposure and burying of each residue of the S1 and S2 peptides in the HLA-DR groove is shown in Fig.4c and 4f respectively. The large binding pocket 1 harbors an aromatic residue Y73 (in S1) or F76 (in S2) making buried aromatic contacts. Pocket 4 is filled either with an aromatic F76 (in S1) or a hydrophobic residue V79 (in S2), while the hydrophobic residues V78 (in S1) and L81 (in S2) are pointing into pocket 6. Although both residues V79 (in S1) and D82 (in S2) bury their side chain in pocket 7, the contribution to the interaction energy of S2 is more of an electrostatic nature via residue 71 of the HLA-DR ß chain, which in most haplotypes is an arginine. The corresponding contribution of L81 (in S1) to the contact area of pocket 9 is less obvious for the S2 peptide were a serine is found. Furthermore, these observations suggest that while the buried peptide residues predominantly contribute to HLA-DR anchoring, the exposed residues mediate both T-cell receptor recognition and the binding to HLA-DR.

EXAMPLE 8

Identification of the minimal epitopes recognized by the region(71-87) T-cell clones

To confirm that both predicted sequences were functional T-cell epitopes, the minimal S1 and S2 11-mer sequences were synthesized and used in proliferation assays with the region(71-87)-specific T-cell clones.

Fig. 2 shows that clones Tc-1 to Tc-6 proliferated after being challenged with the S1 peptide, but not with the S2 peptide. Tc-7 to Tc-10 recognize the S2 peptide but not the S1 peptide. These results indicate
5 that both S1 and S2 are functional T-cell epitopes.

EXAMPLE 9

<u>In silico alanine scan of immunogenic regions</u>

The contribution of each residue to the HLA-DR
10 binding was calculated for both sequences by an <u>in silico</u> alanine scan. The scan of the S1 residues revealed a correlation between the loss of interaction energy and the extent of burying in HLA-DR pockets 1, 4 and 6 (Fig. 4a,c). The residues E75 or E80, respectively at positions
15 3 and 8 in the S1 sequence are not deeply buried in the groove, however an alanine substitution was calculated to result in a substantial loss in interaction energy. The largest contact loss between the S1 peptide and the groove was obtained with a side-chain removal of the
20 residues occupying the pockets 1, 4 or 9 (Fig. 4b). For an alanine substitution of the residues F76, R77 and L81, at positions 1, 2 and 6 respectively in the S2 mode (Fig. 4d-f), an important loss of interaction energy and a proportional loss of contact area was observed. Alanine
25 substitution in pocket 7 of the S2 sequence (D82) revealed no major loss in contact area, however a substantial energy loss was found, which may probably be due to the removal of the ionic interaction with residue R71 of the HLA-DR-ß chain (see also Fig.3b).
30

EXAMPLE 10

<u>Mutagenesis in the immunogenic SakSTAR region 71-87</u>

Altogether, these observations show that mutations at a combination of key positions lead to a
35 reduced immunogenicity of this sequence. Changes into alanine of the important anchoring residues of both epitopes at amino acid positions Y73, F76, V78, and L81 abolished the plasminogen activating potency.

24

Therefore, other residues that were important for T-cell proliferation (Fig. 2) and that contributed to HLA-DR binding were targeted (Fig. 4). Residues K74 and E80 were chosen for their dual contribution in DR-

5   anchoring and facing the TCR in the S1 binding mode. This dual contribution was also found for residue R77 in the S2 binding mode. Furthermore, residues R77 and E80 were found to be solvent exposed at an important TCR recognition position (pocket 5) in the S1 and S2 peptide,

10 respectively. Finally, D82 was targeted for its anchoring contribution in the S2 binding mode.

The SakSTAR-mutants were engineered by the spliced overlap extension polymerase chain reaction (SOE-PCR) (Horton et al., 1989) using as template

15 pMEX.SakSTAR-myc, encoding the wild-type SakSTAR sequence with a carboxy-terminal additional triple alanine followed by a myc sequence (Evan et al., 1985). For each mutant, the amplified product was purified and ligated into the pMEX vector (Schlott et al., 1994). All

20 constructions were confirmed by sequencing of the entire SakSTAR-myc coding region.

The SakSTAR-variants were expressed and purified from transformed E. coli TG1, using experimental conditions as described (Collen et al., 1996a), followed

25 by size exclusion chromatography using a superdex 75 column (Pharmacia, Uppsala, Sweden). The purity of the SakSTAR variants was confirmed by SDS-PAGE to be at least 98%. The following SakSTAR mutants were constructed, SakSTAR(R77A, E80A), SakSTAR(R77A, E80A, D82A) and

30 SakSTAR(K74Q, R77S, E80S, D82S). The specific activity of all these mutants was between 50% and 100% of that of wild-type SakSTAR. Variants of wild-type staphylokinase are identified herein by the substituted amino acids in single letter symbols followed by their position number

35 in the mature SakSTAR sequence (136 amino acids) and by the substituting amino acids in single letter symbols.

EXAMPLE 11

<u>Immunogenicity analysis of the redesigned 71-87 region of</u>
<u>SakSTAR</u>

The S1 and S2 binding mode of the above SakSTAR
5 mutants were remodeled in the HLA-DR binding groove. A
loss of at least 15 kcal/mol in interaction energy and 40
$\mathring{A}^2$ in contact area was found for the S1 peptide of any of
these variants. For the S2 stretch, a loss in interaction
energy was found to be at least 10 kcal/mol, and over 100
10 $\mathring{A}^2$ in the contact area (data not shown), indicating a
reduced binding of the mutant peptide sequences with HLA-
DR.

In addition, the area 69-89 of the SakSTAR
variants were threaded through the binding groove
15 similarly as shown in Fig. 4a to analyze the possibility
of newly introduced epitopes. Importantly, no new binding
motifs in any of the SakSTAR-variants were revealed. All
region(71-87)-specific T-cell clones were tested for
proliferation after challenge with the above described
20 SakSTAR-mutants, and none of them showed proliferation.
This was not due to presentation problems, because T-cell
clones recognizing a different SakSTAR epitope were
perfectly capable to proliferate after stimulation with
either region(71-87) SakSTAR variant.
25

EXAMPLE 12

<u>Cellular immune response to the newly designed molecules</u>

The human T-cells used in the functional assays
were obtained by selection and cloning procedures.
30 Consequently, they may represent only a subset of
region(71-87)-specific T-cells. To investigate whether a
redesigned region actually results in a reduced
immunogenic protein, a cellular immune response to a
redesigned protein should be compared to that of its wild
35 type counterpart.

However, the vast majority of humans does also
recognize additional immunogenic SakSTAR regions
(untouched in these SakSTAR-variants), which would

invalidate such an experiment. Therefore, 25 other
healthy donors were screened for SakSTAR
immunoreactivity. PBMC were thawed, washed four times in
1% human serum albumin, and cultured at 1.8×10^6 cells/ml

5 in RPMI 1640 supplemented with 5% human serum
(Biowitthaker, Walkersville, Maryland) and gentamycin
with or without SakSTAR-myc for 10 days in a humidified
CO_2 incubator. The non-adherent cells were harvested and
restimulated with no antigen, SakSTAR-myc or different

10 peptides. For antigen presenting cells, irradiated
autologous PBMC were T-cell depleted, using CD3-M450
Dynabeads (Dynal, Compiégne, France) according
manufacturer procedures (FACS analysis indicated >95%
non-T-cells). The plates were incubated at 37°C under

15 humidified atmosphere containing 5% CO_2 for 5 days, and
pulsed with BrdU for the last 22 to 24 hours.
         Subsequently, the cells were harvested and
analyzed for their BrdU content (BrdU ELISA, Boehringer
Mannheim, Mannheim, Germany). A stimulation index was

20 calculated, by dividing the OD obtained from the PBMC in
response to an antigen/peptide by that of the spontaneous
proliferation (no antigen) control. A value exceeding 3
was considered positive, indicating the proliferation of
specific T-cells to that particular antigen/peptide.

25   A cellular immune response to the SakSTAR
peptide(71-87) was observed in 80% of the 20 identified
SakSTAR positive donors, confirming its immunodominant
character.
         PBMC from the 10 donors with the highest

30 region(71-87)-specific cellular response were primed with
SakSTAR-myc and subsequently challenged with different
SakSTAR or region-(71-87)-mutant peptides as indicated in
Fig. 5.
         Although the overall cellular response to the

35 single alanine mutated peptides was significantly reduced
as compared to the wild-type peptide (P≤0.05), these
peptides induced a cellular response in several of the
tested donors. The peptides with an alanine at positions

R77 or E80 hampered the lymphocyte proliferation of the
majority of the tested donors. The relative importance of
these residues was also found in the proliferation assays
with the region-(71-87)-specific T-cell clones.

5   However, if SakSTAR-myc primed lymphocytes are
restimulated with the double (R77A, E80A), triple (R77A,
E80A, D82A), quadruple (K74Q, R77S, E80S, D82S) or
quintuple (K74A, E75A, R77A, E80A, D82A) mutated
peptides, none of the tested donors showed a positive
10   cellular immune response (P≤0.001). This implicates that
region(71-87)-specific circulating T-lymphocytes in
humans (which may have been generated by earlier
infections with lysogenic Staphylococcus strains) are no
longer capable of recognizing the mutated region 71-87 of
15   any of the SakSTAR variants. Consequently, their
proliferation and help to support B-lymphocytes to
generate antibodies is impaired.

EXAMPLE 13
20   Identification of critical residues in the other
identified T-cell epitopes in the SakSTAR molecule
           T-cell clones recognizing several of the other
SakSTAR epitopes (see Table I) were analyzed in a similar
manner as the region(71-87)-specific T-cell clones
25   described in the preceding examples. The minimal sequence
requirements for most SakSTAR-specific T-cell clones was
determined in T-cell proliferation assays, using peptides
each containing a different single alanine substitution.
The results are summarized in Figure 6.
30   The T-cell clones that proliferated after a
challenge with the peptide 16-SYFEPTGPYLMVNVTGV-32 could
be divided into two groups, 8 T-cell clones recognized
the epitope 16-26 and 10 clones did recognize the SakSTAR
sequence 23-33. An alanine substitution at F18, or E19
35   abrogated proliferation of all isolated region(16-26)-
specific T-cell clones, whereas most T-cell clones could
no longer proliferate if Y17, or Y24 was mutated into an
alanine. Side-chain removal of P20 or G23 affected the T-

28

cell proliferation of more than half of this group of T-cell clones. All isolated region(23-33)-specific T-cell clones were found sensitive for an alanine substitution at positions Y24, M26, or N28, whereas an alanine
5 substitution of T30 or G31 abrogated proliferation of the majority of the isolated region(16-26)-specific T-cell clones.

Based on these data combined with the calculated residue preference matrices several SakSTAR-
10 mutants were constructed: SakSTAR(P20Y, Y24A), SakSTAR(P20Y, Y24S), SakSTAR(E19D), SakSTAR(Y17L), SakSTAR(F18L), SakSTAR(F18E), SakSTAR(G22S), SakSTAR(G22S, P23G), SakSTAR(N28S), and SakSTAR(V29L, L127V). All these SakSTAR-mutants had a reduced
15 immunogenic profile for the region(16-32) specific T-cell clones.

Analysis of 14 T-cell clones specific for the SakSTAR peptide 56-TKEKIEYYVEWALDATA-72 was performed. An alanine substitution at residues Y62, E65 or W66 resulted
20 in the loss of proliferation of the vast majority of these region-specific T-cell clones. Approximately half of these T-cell clones were found sensitive for side chain removal of residues E61, Y63, V64, L68 or D69. Based on these data combined with the calculated residue
25 preference matrices several SakSTAR-mutants were constructed, however all had lost their thrombolytic activity. The 56-72 immunogenic region of SakSTAR is part of the activation epitope as defined by Jespers et al. Mutation of one of the activation epitope residues into
30 most other residue types creates a biological inactive molecule (Jespers et al (1999)).

Nine T-cell clones specific for the SakSTAR peptide 106-ITEKGFVVPDLSEHIKN-122 were also analyzed. Alanine substitution at positions P114, L116, E118, or
35 H119 abolished proliferation of the majority of the region(106-122)-specific T-cell clones. Side chain removal of F111 or D115 affected the proliferative capacity of approximately half of these T-cell clones.

29

Based on these data combined with the calculated residue
preference matrices several SakSTAR-mutants were
constructed: SakSTAR(H119A), SakSTAR(H119S),
SakSTAR(V89L, L116Y), SakSTAR(V89L, L116T),
5 SakSTAR(V112T), SakSTAR(V112S), SakSTAR(D115N), and
SakSTAR(E118S). All these SakSTAR-mutants had a
significantly reduced immunogenic profile for the
region(106-122) specific T-cell clones. Although an
alanine change at V112 had an effect of only 11% of the
10 region-specific T-cell clones, interestingly the
biologically active mutants SakSTAR(V112T) and
SakSTAR(V112S) could not induce proliferation in any of
the region(106-122)-specific T-cell clones. This was not
due to the construction (as confirmed by sequencing) or
15 purity of the isolated SakSTAR mutant, because T-cell
proliferation of other SakSTAR-specific T-cell clones
could be induced using these mutants comparable to that
with wild-type SakSTAR.

        The following combinatorial mutants have been
20 constructed and isolated; SakSTAR(V112T, H119A),
SakSTAR(V112T, H119S), SakSTAR(V112T, E118S, H119S), and
SakSTAR(V112T, H119S, K130Y). These thrombolytically
active mutants lost their capacity to induce the
region(106-122)-specific T-cell clone proliferation,
25 indicating that the immunogenicity of SakSTAR region 106-
122 was eliminated.

        Finally, 7 T-cell clones recognizing the
SakSTAR peptide 120-IKNPGFNLITKVVIEKK-136 were analyzed
for their minimal sequence requirements. Alanine
30 substitution at positions G124, F125, N126, or L127
abolished proliferation of the majority of the
region(120-136)-specific T-cell clones. Side chain
removal of I128, T129, K130, V131, or V132 affected the
proliferative capacity of approximately half of these T-
35 cell clones. Based on these data combined with the
calculated residue preference matrices, two biologically
active SakSTAR-mutants were constructed. The mutant
SakSTAR(R77E, E134R) has been tested in proliferation

assays using several region(120-136)-specific T-cell
clones and some region(71-89)-specific T-cell clones. It
was found that this mutant could support the
proliferation of some, but not all T-cell clones from
5 both specificities, indicating that two SakSTAR regions
had a reduced immunogenic profile. Although the mutation
of residue K130 into alanine in the SakSTAR120-136
peptide did only have an effect on approximately half of
the isolated region(120-136)-specific T-cell clones, this
10 residue was targeted. Computer modeling supplemented with
the residue preference matrix revealed that if an Y
replaces residue K130 within this epitope (GFLIT_YVVIE),
the interaction energy between this mutant-epitope and
the HLA-DR binding groove is so high, that binding within
15 the groove is predicted to be impossible. Therefore,
mutant SakSTAR(K130Y) was constructed, expressed and
purified, and was found to have a thrombolytic activity
similar to that of wild-type SakSTAR. The region(120-
136)-specific T-cell clones were tested with this
20 SakSTAR-mutant, and none of them could proliferate,
suggesting the epitope was no longer functional.
Therefore, mutation of residue K130 of SakSTAR into Y is
the ultimate mutation, because the presentation of this
redesigned region is completely lost.

25

Altogether, these analyses demonstrate that the
immunogenicity of a heterologous protein can be reduced
by the removal of functional T-cell epitopes. As a
consequence, the elimination of a combination of
30 immunogenic regions will further reduce the T-cell
reactivity, and therefore the overall immunogenicity of a
combined mutant protein. Furthermore, the rational
approach combining functional assays with mathematical
models may lead to the precise removal of HLA-DR
35 anchoring residues, affecting the presentation of T-cell
epitopes and the initiation of T-cell reactivity.

REFERENCES

Behnke, D., and Gerlach, D. (1987). Cloning and
expression in Escherichia coli, Bacillus subtilis, and
Streptococcus sanguis of a gene for staphylokinase--a
5 bacterial plasminogen activator. Mol Gen Genet <u>210</u>, 528-
534.

Brooks, B. R., Bruccoleri, R., Olafson, D., States, D.
J., Swaminathan, S., and Karplus, M. (1983). CHARMM: A
10 Program for macromolecular energy, minimization, and
dynamics calculations. J. Comput. Chem <u>4</u>, 187-217.

Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L.
J., Urban, R. G., Strominger, J. L., and Wiley, D. C.
15 (1993). Three-dimensional structure of the human class II
histocompatibility antigen HLA-DR1. Nature <u>364</u>, 33-39.

Buyse, I., Decorte, R., Baens, M., Cuppens, H., Semana,
G., Emonds, M. P., Marynen, P., and Cassiman, J. J.
20 (1993). Rapid DNA typing of class II HLA antigens using
the polymerase chain reaction and reverse dot blot
hybridization. Tissue Antigens <u>41</u>, 1-14.

Collen, D. (1998). Staphylokinase: a potent, uniquely
25 fibrin-selective thrombolytic agent. Nat Med <u>4</u>, 279-284.

Collen, D., Bernaerts, R., Declerck, P., De Cock, F.,
Demarsin, E., Jenne, S., Laroche, Y., Lijnen, H. R.,
Silence, K., and Verstreken, M. (1996a). Recombinant
30 staphylokinase variants with altered immunoreactivity. I:
Construction and characterization. Circulation <u>94</u>, 197-
206.

Collen, D., Moreau, H., Stockx, L., and Vanderschueren,
35 S. (1996b). Recombinant staphylokinase variants with
altered immunoreactivity. II: Thrombolytic properties and
antibody induction. Circulation <u>94</u>, 207-216.

32

Collen, D., Zhao, Z. A., Holvoet, P., and Marynen, P. (1992). Primary structure and gene structure of staphylokinase. Fibrinolysis 6, 226-231.

5 De Maeyer, M., Desmet, J., and Lasters, I. (1997). All in one: a highly detailed rotamer library improves both accuracy and speed in the modelling of side chains by dead-end elimination. Fold Des 2, 53-66.

10 Delhaise, P., Bardiaux, M., and Wodak, S. (1984). Interactive computer animation of macromolecules. J Mol Graphics 2, 103-106.

Desmet, J., De Maeyer, M., Hazes, B., and Lasters, I.
15 (1992). The dead-end elimination theorem and its use in protein side-chain positioning. Nature 356, 539-542.

Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985). Isolation of monoclonal antibodies specific for
20 human c-myc proto-oncogene product. Mol Cell Biol 5, 3610-3616.

Hiemstra, H. S., Duinkerken, G., Benckhuijsen, W. E., Amons, R., de Vries, R. R., Roep, B. O., and Drijfhout,
25 J. W. (1997). The identification of CD4+ T-cell epitopes with dedicated synthetic peptide libraries. Proc Natl Acad Sci U S A 94, 10313-10318.

Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and
30 Pease, L. R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77, 61-68.

Jespers, L., Vanwetswinkel, S., Lijnen, R., Van Herzeele,
35 N., Van Hoef, B., Demarsin, E., Collen, D., and De Maeyer, M. (1999). Structural and functional basis of plasminogen activation by staphylokinase. Thrombosis Hemostasis 81, 479-485.

Koradi, R., Billeter, M., and Wuthrich, K. (1996).
MOLMOL: a program for display and analysis of
macromolecular structures. J Mol Graphics 14, 51-55.

5 Miller, G., and Lipman, M. (1973). Release of infectious
Epstein-Barr virus by transformed marmoset leukocytes.
Proc Natl Acad Sci U S A 70, 190-194.

Sako, T., and Tsuchida, N. (1983). Nucleotide sequence of
10 the staphylokinase gene from Staphylococcus aureus.
Nucleic Acids Res 11, 7679-7693.

Schlott, B., Hartmann, M., Guhrs, K. H., Birch-
Hirschfeid, E., Pohl, H. D., Vanderschueren, S., Van de
15 Werf, F., Michoel, A., Collen, D., and Behnke, D. (1994).
High yield production and purification of recombinant
staphylokinase for thrombolytic therapy. Biotechnology (N
Y) 12, 185-189.

20 Warshel, A., and Levitt, M. (1976). Theoretical studies
of enzymic reactions: dielectric, electrostatic and
steric stabilization of the carbonium ion in the reaction
of lysozyme. J Mol Biol 103, 227-249.

25 Wodak, S. J., De Coen, J. L., Edelstein, S. J., Demarne,
H., and Beuzard, Y. (1986). Modification of human
hemoglobin by glutathione. III. Perturbations of
hemoglobin conformation analyzed by computer modeling. J
Biol Chem 261, 14717-14724.

CLAIMS

1. Method for reducing the immunogenicity of a peptide or protein, which method comprises the steps of:

a) designing a series of overlapping test peptides each having an amino acid sequence that corresponds with part of the amino acid sequence of the peptide or protein of which the immunogenicity is to be reduced;

b) identifying which of the test peptides of the series comprises one or more T-cell epitope(s);

c) modifying the amino acid sequence of one or more of the test peptides comprising one or more T-cell epitope(s);

d) repeating step b) and optionally step c) with the modified test peptides until one or more of the T-cell epitopes originally comprised therein are significantly reduced or eliminated;

e) modifying the amino acid sequence of the peptide or protein of which the immunogenicity is to be reduced according to the T-cell eliminating modifications made in the amino acid sequence of the test peptides to produce a modified peptide or protein.

2. Method as claimed in claim 1, wherein the identification of test peptides comprising a T-cell epitope is performed by means of a functional T-cell assay in which proliferation of one or more T-cell clones after stimulation of the T-cell clone(s) with one of the test peptides means that the said test peptide comprises a T-cell epitope.

3. Method as claimed in claim 1, wherein the identification of test peptides comprising a T-cell epitope is performed by means of a functional T-cell assay in which proliferation of one or more T-lymphocytes as present in the circulation of humans (PBMC) after stimulation of the PBMC with one of the test peptides means that the said test peptide comprises a T-cell epitope.

4. Method as claimed in claim 1, wherein the identification of test peptides comprising a T-cell epitope is performed by determining the interaction energy of the test peptide with the binding groove of one or more HLA-DR haplotypes by means of computer modeling, in which test peptide(s) are identified as comprising a T-cell epitope when according to their interaction energy they fit into the HLA-DR binding groove.

5. Method as claimed in claim 1, wherein the identification of test peptides comprising a T-cell epitope is performed by a combination of the following tests:

a) a functional T-cell assay in which proliferation of one or more T-cell clones after stimulation of the T-cell clone(s) with one of the test peptides means that the said test peptide comprises a potential T-cell epitope; and/or

b) a functional T-cell assay in which proliferation of one or more T-lymphocytes as present in the circulation of humans (PBMC) after stimulation of the PBMC with one of the test peptides means that the said test peptide comprises a T-cell epitope; and

c) determining the interaction energy of the said test peptide with the binding groove of one or more HLA-DR haplotypes by means of computer modeling, in which the test peptide is identified as comprising a potential T-cell epitope when according to its interaction energy it fits into the HLA-DR binding groove, wherein a test peptide is considered to comprise a T-cell epitope if it is identified in test a) and/or b) and c).

6. Method as claimed in claims 1-5, further comprising one or more repetitions of steps b) to e) with the modified peptide or protein instead of the test peptides.

7. Method as claimed in claims 1-6, which method comprises the steps of:

a) designing a series of overlapping test peptides each having an amino acid sequence that

corresponds with part of the amino acid sequence of the peptide or protein of which the immunogenicity is to be reduced;

b) identifying which of the test peptides of
5 the series comprises one or more T-cell epitope(s);

c) modifying the amino acid sequence of one or more of the test peptides comprising one or more T-cell epitope(s);

d) testing the modified peptides for their
10 potential to activate T-cells and induce proliferation of either T-cell clones or T-lymphocytes as present in the circulation of humans (PBMC);

e) modifying the amino acid sequence of the peptide or protein of which the immunogenicity is to be
15 reduced according to the T-cell eliminating modifications made in the amino acid sequence of the test peptides;

f) construction and purification of the modified peptide or protein of which the immunogenicity is to be reduced in a suitable host, and testing its
20 biological activity;

g) repeating step c-f) with the modified test peptides or protein until one or more of the T-cell epitopes originally comprised therein are significantly reduced or eliminated and contemplating the biological
25 activity.

8. Method for reducing the immunogenicity of a peptide or protein, which method comprises the steps of:

a) designing a series of overlapping test peptides each having an amino acid sequence that
30 corresponds with part of the amino acid sequence of the peptide or protein of which the immunogenicity is to be reduced;

b) identifying which of the test peptides of the series comprises one or more T-cell epitope(s);

35 c) modifying the amino acid sequence of one or more of the test peptides comprising one or more T-cell epitope(s);

d) testing the modified peptides for their potential to activate T-cells and induce proliferation of either T-cell clones or T-lymphocytes as present in the circulation of humans (PBMC);

5   e) modifying the amino acid sequence of the peptide or protein of which the immunogenicity is to be reduced according to the T-cell eliminating modifications made in the amino acid sequence of the test peptides;

f) construction and purification of the
10   modified peptide or protein of which the immunogenicity is to be reduced in a suitable host, and testing its biological activity;

g) repeating step c-f) with the modified test peptides or protein until one or more of the T-cell
15   epitopes originally comprised therein are significantly reduced or eliminated and contemplating the biological activity.

9. Method as claimed in claim 7 and 8, wherein the step of identification of which of the test peptides
20   of the series comprises one or more T-cell epitope(s) is performed as described in claims 2, 3 or 4.

10. Method as claimed in claims 1-9, wherein the T-cell epitopes are eliminated by minimal residue/region mutagenesis.

25   11. Method as claimed in claims 1-10, wherein the T-cell epitopes are eliminated by deletion or insertion of residues in the identified T-cell epitopes.

12. Method as claimed in claims 1-11, wherein the T-cell epitopes are eliminated by substitution with
30   human sequences, or human homologous sequences.

13. Method as claimed in claims 1-12, wherein the T-cell epitopes are eliminated by enhancing the sensitivity of the identified T-cell epitopes for proteolytic endosomal enzymes.

35   14. Method as claimed in claims 1-13, further comprising the identification and removal of HLA-DR anchoring residues of the peptide or protein to reduce the immunogenicity thereof.

15. Method as claimed in claims 1-14, wherein the biological activity of the protein or peptide with reduced immunogenicity is tested after modification of the amino acid sequence of the protein or peptide.

5   16. Method for producing a peptide or protein having a reduced immunogenicity in comparison to the wild type peptide or protein, comprising the following steps:

a) providing a DNA sequence encoding at least the amino acid sequence of the peptide or protein that

10 provides for the biological activity thereof;

b) identifying one or more of the T-cell epitopes comprised in the amino acid sequence of the wild type peptide or protein by:

1) designing a series of overlapping test

15 peptides each having an amino acid sequence that corresponds with part of the amino acid sequence of the peptide or protein;

2) identifying which of the test peptides of the series comprises one or more T-cell epitope(s);

20   3) modifying the amino acid sequence of one or more of the test peptides comprising one or more T-cell epitope(s);

4) repeating step 2) and optionally step 3) with the modified test peptides until one or more of

25 the T-cell epitopes originally comprised therein are significantly reduced or eliminated;

c) modifying the DNA sequence to introduce the modifications to the T-cell epitope(s) in the amino acid sequence encoded thereby;

30   d) introducing the DNA sequence into a host organism;

e) culturing the host organism under conditions allowing expression of the modified peptide or protein; and

35   f) isolating the peptide or protein having a reduced immunogenicity from the host organism.

17. Method for producing a peptide or protein having a reduced immunogenicity in comparison to the wild type peptide or protein, comprising the following steps:

a) providing a DNA sequence encoding at least
5 the amino acid sequence of the peptide or protein that provides for the biological activity thereof;

b) identifying and modifying one or more of the T-cell epitopes comprised in the amino acid sequence of the wild type peptide or protein by:

10   1) identifying which of the test peptides of the series comprises one or more T-cell epitope(s);

2) modifying the amino acid sequence of one or more of the test peptides comprising one or more T-cell epitope(s);

15   3) testing the modified peptides for their potential to activate T-cells and induce proliferation of either T-cell clones or T-lymphocytes as present in the circulation of humans (PBMC);

4) modifying the amino acid sequence of
20 the peptide or protein of which the immunogenicity is to be reduced according to the T-cell eliminating modifications made in the amino acid sequence of the test peptides;

5) construction and purification of the
25 modified peptide or protein of which the immunogenicity is to be reduced in a suitable host, and testing its biological activity;

6) repeating step 2-5) with the modified test peptides or protein until one or more of the T-cell
30 epitopes originally comprised therein are significantly reduced or eliminated and contemplating the biological activity;

c) modifying the DNA sequence to introduce the modifications to the T-cell epitope(s) in the amino acid
35 sequence encoded thereby;

d) introducing the DNA sequence into a host organism;

e) culturing the host organism under conditions allowing expression of the modified peptide or protein; and

f) isolating the peptide or protein having a
5 reduced immunogenicity from the host organism.

18. DNA construct encoding the modified DNA sequence as defined in claim 16 or 17.

19. Host organism, comprising the DNA construct as claimed in claim 18.

10 20. Modified peptide or protein showing a reduced T-cell based immunogenicity as compared to the wild type peptide or protein and differing from the wild type peptide or protein in at least one amino acid substitution, deletion or insertion that leads to the
15 elimination of at least one T-cell epitope but does not lead to loss of the biological activity of the peptide or protein.

21. Modified peptide or protein as claimed in claim 20, obtainable by means of the method as claimed in
20 claims 1-17.

22. Modified peptide or protein as claimed in claims 20 and 21 for use in treatment, diagnosis or prophylaxis.

23. Use of a modified peptide or protein as
25 claimed in claims 20 and 21 for the preparation of a pharmaceutical composition for treatment, diagnosis or prophylaxis of a human subject.

24. Pharmaceutical composition comprising a modified peptide or protein as claimed in claims 20 and
30 21 together with a pharmaceutically acceptable carrier, diluents or excipient.

25. Modified peptide or protein as claimed in claim 20 or 21, which protein is staphylokinase.

26. Staphylokinase variants in which one or
35 more of the following immunogenic regions are modified such that the T cell epitopes contained therein are eliminated:

1-SSSFDKGKYKKGDDASY-17,

41

16-SYFEPTGPYLMVNVTGV-32,

56-TKEKIEYYVEWALDATA-72,

71-TAYKEFRVVELDPSAKI-87,

106-ITEKGFVVPDLSEHIKN-122, and

5   120-IKNPGFNLITKVVIEKK-136.

27. Staphylokinase variants as claimed in claim 26 with reduced T-cell reactivity as compared to wild type staphylokinase, but retaining its biological activity, which variants have the amino acid sequence 10 depicted in Figure 1 with one or more of the following amino acid substitutions in one or more of the following immunogenic region:

a) in the immunogenic region comprising SakSTAR residues 16-32 the substitutions Y17L; F18L; F18E; E19D; 15 P20Y, Y24A; P20Y, Y24S; G22S; G22P, P23G; or N28S;

b) in immunogenic region comprising SakSTAR residues 71-82 the substitutions F76W, V79Y, D82A; R77A, E80A; R77S, E80S; R77A, E80A, D82A; K74Q, R77S, E80S; K74Q, R77S, E80S, D82G; K74Q, R77S, E80S, D82A; K74Q, 20 R77S, E80A, D82A; K74Q, R77A, E80S, D82A; K74Q, R77S, E80S, D82S; or V79Y, D82A;

c) in immunogenic region comprising SakSTAR residues 106-122 the substitutions V112T; V112S; D115N; E118S; H119A; H119S; V89L, L116Y; V89L, L116T; 25 V112T, H119S; V112T, H119A; or V112T, E118S, H119S;

d) in immunogenic region comprising SakSTAR residues 120-136, the substitution K130Y.

28. Staphylokinase variants as claimed in claim 27, having a combination of one or more of the mutations 30 listed in claim 27.

29. Staphylokinase variants as claimed in claim 28, having one of the following combination of mutations V112T, H119S, K130Y; R77E, E134R; V29L, L127V; K74Q, R77E, E80S, D82S, E134R; R77S, E80S, V112T, H119S; R77S, 35 E80S, V112T, H119S, K130Y; R77A, E80A, V112T, H119S; R77A, E80A, V112T, H119S, K130Y; K74Q, R77S, E80S, V112T, H119S; K74Q, R77S, E80S, V112T, H119S, K130Y; K74Q, R77S,

E80S, D82S, V112T, H119S; or K74Q, R77S, E80S, D82S,
V112T, H119S, K130Y.

30. Staphylokinase variant selected from the
group consisting of SakSTAR(P20Y, Y24A), SakSTAR(P20Y,
5 Y24S), SakSTAR(E19D), SakSTAR(Y17L), SakSTAR(F18L),
SakSTAR(F18E), SakSTAR(G22S), SakSTAR(G22S, P23G),
SakSTAR(N28S), SakSTAR(V29L, L127V), SakSTAR(R77E,
E134R), SakSTAR(K74Q, R77E, E80S, D82S, E134R),
sakSTAR(K74Q, R77E, E80S, D82S,V112T, H119S, E134R),
10 sakSTAR(K74Q, R77E, E80S, D82S,V112T, H119S, E134R,
K130Y), SakSTAR(R77S, E80S, V112T, H119S), SakSTAR(R77S,
E80S, V112T, H119S, K130Y), SakSTAR(R77A, E80A, V112T,
H119S), SakSTAR(R77A, E80A, V112T, H119S, K130Y),
SakSTAR(K74Q, R77S, E80S, V112T, H119S), SakSTAR(K74Q,
15 R77S, E80S, V112T, H119S, K130Y), SakSTAR(K74Q, R77S,
E80S, D82S, V112T, H119S), SakSTAR(K74Q, R77S, E80S,
D82S, V112T, H119S, K130Y), SakSTAR(V112T, H119A),
SakSTAR(V112T, H119S), SakSTAR(V112T, E118S, H119S),
SakSTAR(V112T, H119S, K130Y), SakSTAR(V112T),
20 SakSTAR(V112S), SakSTAR(H119A), SakSTAR(H119S),
SakSTAR(V89L, L116Y), SakSTAR(V89L, L116T),
SakSTAR(V112T), SakSTAR(V112S), SakSTAR(D115N),
SakSTAR(E118S), SakSTAR(K130Y).

31. Staphylokinase variant as claimed in claims
25 25-30 for use in treatment, diagnosis or prophylaxis.

32. Use of a staphylokinase variant as claimed
in claims 25-30 for the preparation of a pharmaceutical
composition for treatment, diagnosis or prophylaxis of a
human subject.

30   33. Pharmaceutical composition comprising a
staphylokinase variant as claimed in claims 25-30
together with a pharmaceutically acceptable carrier,
diluents or excipient.

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```
 1        10         20          30          40          50
 |         |          |           |           |           |
SSSFDKGKYK KGDDASYFEP TGPYLMVNVT GVDSKGNELL SPHYVEFPIK

          60         70          80          90         100
           |          |           |           |           |
PGTTLTKEKI EYYVEWALDA TAYKEFRVVE LDPSAKIEVT YYDKNKKKEE

         110        120         130        136
           |          |           |          |
TKSFPITEKG FVVPDLSEHI KNPGFNLITK VVIEKK
```
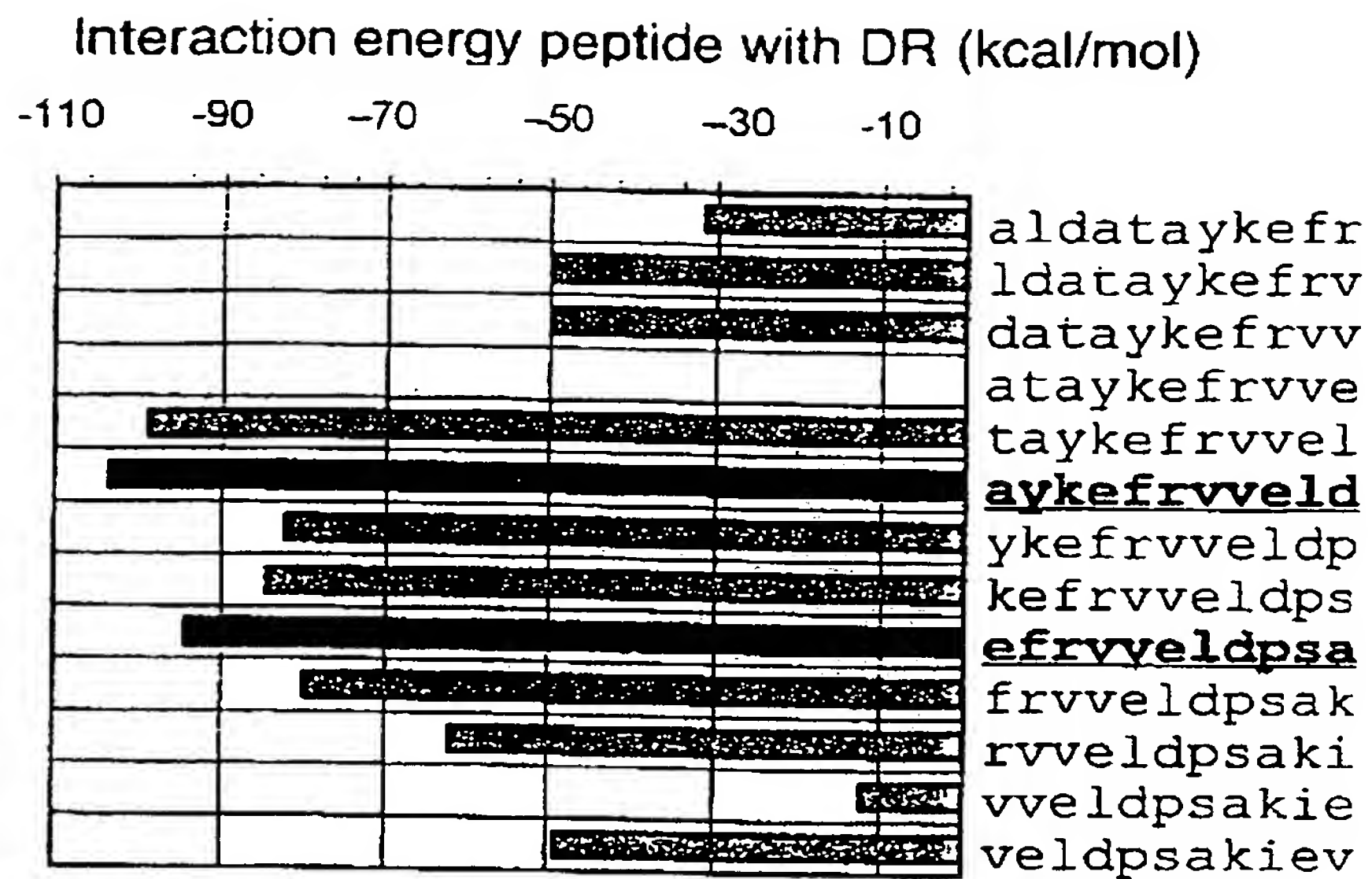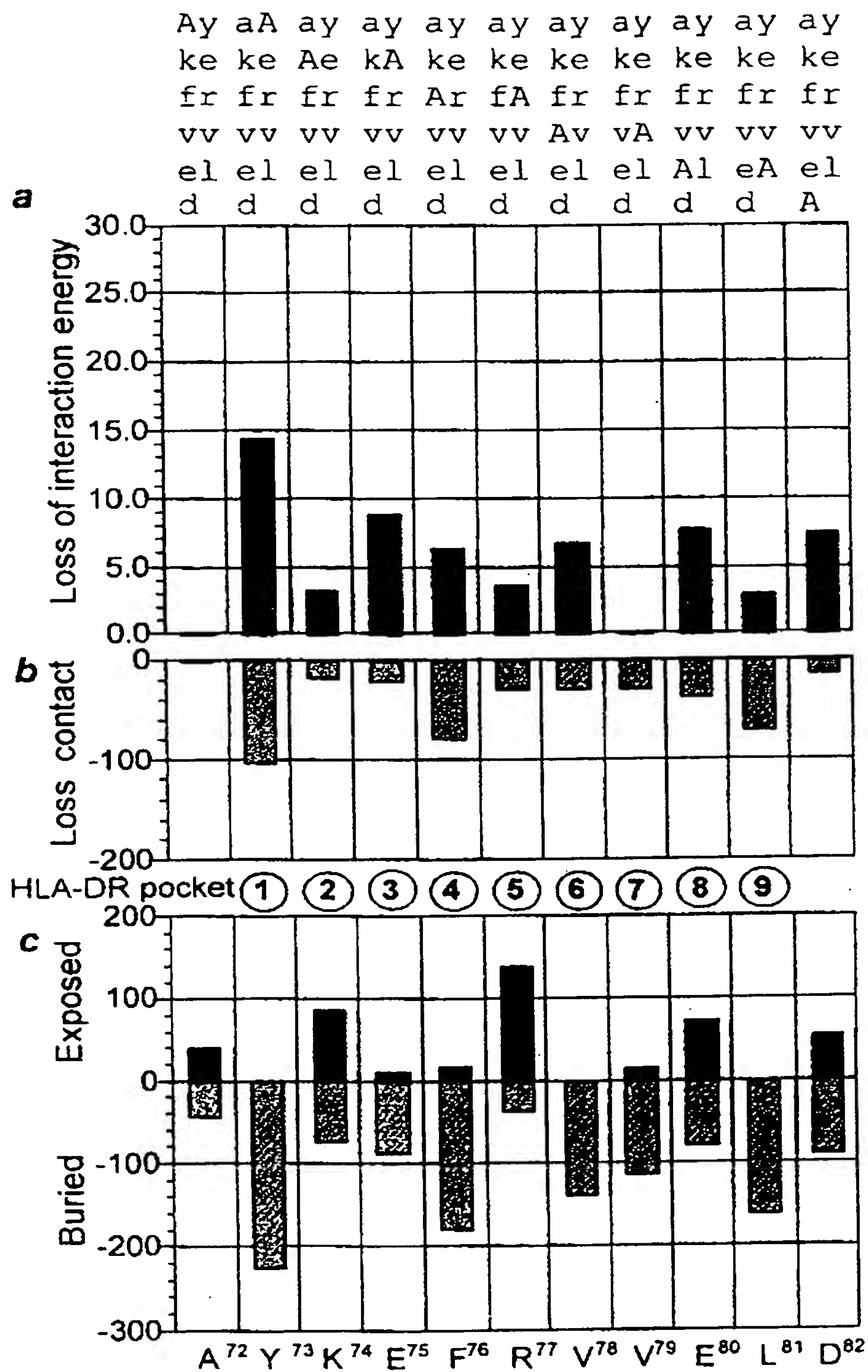
FIG. 1

Peptides

Single alanine mutated region-(71-87) peptides

T cell clones	71TAYKEFRVVELDPSAKI87	AAYKEFRVVELDPSAKI	TAAKEFRVVELDPSAKI	TAYAEFRVVELDPSAKI	TAYKAFRVVELDPSAKI	TAYKEARVVELDPSAKI	TAYKEFAVVELDPSAKI	TAYKEFRAVELDPSAKI	TAYKEFRVAELDPSAKI	TAYKEFRVVALDPSAKI	TAYKEFRVVEADPSAKI	TAYKEFRVVELAPSAKI	TAYKEFRVVELDASAKI	TAYKEFRVVELDPAAKI	TAYKEFRVVELDPSAAI	TAYKEFRVVELDPSAKA	S1 72AYKEFRVVELD82	S2 73EFRVVELDPSA85
Tc-1																		
Tc-2																		
Tc-3																		
Tc-4																		
Tc-5																		
Tc-6																		
Tc-7																		
Tc-8																		
Tc-9																		
Tc-10																		

FIG. 2

Interaction energy peptide with DR (kcal/mol)

-110   -90   -70   -50   -30   -10

aldataykefr
ldataykefrv
dataykefrvv
ataykefrvve
taykefrvvel
aykefrvveld
ykefrvveldp
kefrvveldps
efrvveldpsa
frvveldpsak
rvveldpsaki
vveldpsakie
veldpsakiev

FIG. 3A

FIG. 3B

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FIG. 4A

FIG. 4B

FIG. 5

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FIG. 6